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ANTI-INFLAMMATORY COMPOSITIONS

Abstract:

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1390542 Anti-inflammatory compositions OTSUKA KAGAKU YAKUHIN KK 10 April 1972 [14 April 1971] 16386/72 Heading A5B [Also in Division C3] An anti-inflammatory composition comp- rises (a) a water-insoluble modified protease consisting of a protease bonded to a poly- mer of 20,000-200,000 molecular weight which is a poly (amino acid), a polysacchar- ide, polyaminostyrene or a copolymer of ethylene and maleic acid or anhydride and (b) a carrier, there being no water-soluble protease present. Poly (amino acids) specified include a copolymer of L-leucine and p-amino -D, L-phenyl alanine, poly (N-carboxy-[gamma]- methyl-glutamic anhydride), poly (N-carboxy-[gamma]-methyl-tyrosine anhydride) and poly (N-carboxy-tyrosine anhydride). Polysaccha- rides specified include cellulose, diethylamino- ethyl cellulose, p-amino benzyl cellulose, m- aminoanisole cellulose, m-aminobenzoyloxy- methyl cellulose, 3-(p-amino-m-methylanilino-5-chloro)-1-triacetyno cellulose, bromoacetyl cellulose, cellulose citrate, dextran, diethyl- aminoethyl dextran and dextran isocyanate. Proteases specified include trypsin, pepsin, chymotrypsin, pancreatin, bromelin, papain, and those derived from the microorganisms: Aspergillus melleus, Aspergillus oryzae, Penicillium notatum, Rhizopus chinensis, Mucor racemosis, Trametes sanguinea, Llodella subpiliata, Bacillus subtilis, Pseudomonas myxogene, Streptomyces griseus Streptomyces fradiase. The pro- tease may be modified by reaction with the monomer of the desired polymer. Data supplied from the esp@cenet database - Worldwide

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(54) ANTI-INFLAMMATORY COMPOSITIONS

(71) We, OTSUKA KAGAKU YAKUHIN KABUSHIKI KAISHA, a Corporation organized and existing under the laws of Japan, of No. 10, Bungomachi, Higashi-ku, Osaki-shi, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to an anti-inflammatory composition.

It is well known in the art that proteases have an anti-inflammatory activity. However, the proteases can not be used in doses sufficient to produce satisfactory anti-inflammatory activities, since they display considerable harmful side effects at the same time. For instance, when the protease is administered locally the antibody substance will be produced, and when administered orally it will stimulate the mucous membrance of intestine due to a high order of proteolytic activity thereof. Indeed, the present inventors conducted inhibition tests on edema caused by carrageenin using various proteases through intraperitoneal administration, the most usual method of administration employed at present, whereby it was found that although each of the proteases produced an appreciable inhibitive effect on edema, an increased dosage for improved effect caused bleeding in the abdominal cavity or ascites. This indicates that each protease has to be administered within a permissible range of dosage to prevent such harmful side effects. Such tendency was found with respect to various proteases. Examples of the proteases are trypsin, chymotrypsin and the like from animal sources, bromelin, papain and the like from vegetable sources; and those obtained from microorganisms such as Streptomyces griseus, Bacillus subtilis, Aspergillus melleus, Aspergillus niger and other bacteria of Genus Serratia and Genus Bacillus.

The anti-inflammatory agent of the invention comprises a water-insoluble modified protease and an adjuvant, said water-insoluble modified protease being a protease chemically bonded with a high molecular weight substance, and said high molecular weight substance having a molecular weight of from 20,000 to 200,000 and being selected from the group consisting of amino acid polymers having a skeletal chain containing repetitive peptide bonds, polysaccharides, polyaminostyrenes and copolymers of ethylene and maleic acid or anhydride, there being no water soluble protease or water soluble modified protease present.

In the prior art it has been considered essential that proteases must have a high order of proteolytic activity and be water-soluble in order to display anti-inflammatory activity. This is, for example, due to the belief that the protease rendered water-insoluble by modification will never display any anti-inflammatory effect through oral or local administration, since such modified protease will have extremely low or no proteolytic activity and be no longer absorbed from intestines. However, the researches of the present inventors revealed that when proteases are chemically bonded with a high molecular weight substance to produce water-insoluble modified proteases having much lower proteolytic activity than that of the original proteases, such modified proteases still retain sufficient anti-inflammatory activity and are completely free from harmful side effects. The reason why the above results can be obtained by such modified protease has not been made clear yet, but it has been ascertained by the present inventors that the esterolytic activity of the protease is not reduced so eminently as proteolytic activity by the modification and that anti-inflammatory effect can be ensured as far as the modified protease has esterolytic activity even though it has a low order of proteolytic activity and is water-insoluble. Moreover, the modified protease of the invention can be administered orally or locally without any harmful side effect, since it is not likely to be absorbed from the intestine due to water-insolubility thereof and will hardly stimulate mucous membrane nor produce anti-

body substance due to low proteolytic activity thereof.

The protease to be modified in accordance with the present invention includes various proteases obtained from animals, vegetables and microorganisms. Examples are those of animal sources such as trypsin, pepsin, chymotrypsin and pancreatin; those of vegetable sources such as bromelin and papain; those from microorganisms such as filamentous fungus, basidiomycetes, bacterium and ray fungus. Proteases obtained from filamentous fungus are, for example, those from Aspergillus melleus, Aspergillus oryzae, Penicillin notatum, Rhizopus chenensis and Mucor racemosus. Proteases from basidomycetes are those from Trametes sanguinea and Llodella subpileata. Proteases from bacterium are those from Bacillus subtilis and Pseudomonas myxogenes. Proteases from ray fungus are those from Streptomyces griseus and Streptomyces fradiase. Among these proteases chymotrypsin, trypsin and proteases from Bacillus subtilis and Aspergillus melleus are preferred.

The high molecular weight substances used in the invention are those having a molecular weight of from 20,000 to 200,000 and are 1) amino acid polymers having a skeletal chain containing repetitive peptide bonds, 2) polysaccharides, 3) polyaminostyrenes or 4) copolymers of ethylene and maleic acid or anhydride thereof. Of these particularly preferable are amino acid polymers and polysaccharides. Preferable examples of amino acid polymers are a copolymer of L-leucine and p-amino-DL-phenyl aniline, a polymer of N - carboxy - γ - methyl - glutamic anhydride, a polymer of N - carboxy - γ - methyl tryosine anhydride, and a polymer of N-carboxy tyrosine anhydride. There may be used substances which produce the above polymers during the reaction with protease, such as N-carboxy - γ - methyl - glutamic anhydride, N - carboxy - γ - methyl tyrosine anhydride, or N-carboxy tyrosine anhydride. Preferable polysaccharides are, for example, cellulose and derivatives thereof such as diethylaminoethyl cellulose, carboxymethyl cellulose, p-aminobenzyl cellulose, m-aminoanisole cellulose, m-aminobenzyloxymethyl cellulose, 3-(p-amino-m - methylanilino - 5 - chloro) - 1 - triacetyno cellulose, carboxymethyl cellulose azide, cyanur cellulose, bromoacetyl cellulose and cellulose citrate, and dextran and derivatives thereof such as diethylaminoethyl dextran, carboxymethyl dextran, reaction product of dextran and isocyanate.

Of these high molecular weight substances those having a reactivity with protease by covalent bond or ionic bond to produce water-insoluble protease are used for the modification of protease as they are. For example, carboxymethyl cellulose azide, cyanur cellulose, bromoacetyl cellulose and the reaction product of dextran and isocyanate can be reacted and bonded by covalent bond with protease to produce water-insoluble protease. Further, those having a carboxyl group or diethylaminoethyl group in the molecule can be reacted and bonded with protease by ionic bond to produce water-insoluble protease. In the case of high molecular weight substances having no reactivity with proteases to produce water-insoluble proteases, on the other hand, they are reacted with proteases after a group reactive with protease, such as $-\overset{+}{N}\equiv N$, $-N=\overset{+}{N}=N^-$, COCl, —NCNH, —NCO, —CN, a halogen or an anion or cation exchange group is introduced thereto in conventinal manner. For example, $-\overset{+}{N}\equiv N$ bond is introduced into the molecule of the high molecular weight substance having an amino group by diazotization thereof in conventional manner. Further, $-N=\overset{+}{N}=N^-$, COCl, —NCNH, —NCO or —CN group can be introduced into the molecule of the high molecular weight substance having a carboxyl group by reacting it with azides, chlorides, carbodiimides, isocyanates or cyanes in conventional manner. A halogen atom can be introduced into the molecule of the high molecular weight substances having for example a hydroxyl group or carboxyl group in a conventional manner. An anion or cation exchange group can also be introduced thereto in a conventional manner.

The modification of protease with the above high molecular weight substances is known in the art and according to the invention such modification can be carried out in a conventional manner. For example, the modification with the high molecular weight substance having $-\overset{+}{N}\equiv N$ in the molecule is carried out by coupling it with protease. This reaction is disclosed, for example, in "Naturwiss." Vol. 40, 508 (1953) by N. Grubhofer et al, in "J. Biol. Chem." Vol. 237, 1832 (1962) by A. N. Glazer, et al, in USP 3,167,485 by E. Katchalski, in "Biochemistry" Vol. 3, 1913 (1964) by L. Goldstein et al and in "Biochem. J." Vol. 95, No. 3, 45—46 (1965) by M. Dilly et al. The high molecular weight substance having $-N_3$, —COCl, —NCNH, —NCO or —CN in the molecule is chemically bonded with protease by reacting such groups with amino groups of protease to produce modified protease. This modification is disclosed, for example, in "Seventeenth Collection of Lecture at Enzyme Chemistry Symposium", P 21 (1965) by Toru Takima et al, staged at Tobushima-ken, Japan, in Japanese Patent Publication No. 27492/1964 by Jiro Kirimura et al and in "Biochemistry" Vol. 3, 1905 (1964) by Y. Lerin et al. The protease can be modified by reaction with

the high molecular weight substance having a halogen atom in the molecule. This modification method is disclosed, for example, in "J. Am. Chem. Soc." Vol. 69, 1551 (1947) by R. B. Woodward et al and in "J. Biol. Chem." Vol. 236, 1720 (1961) by J. J. Cebra et al. The modified protease can also be prepared by bonding proteases with the high molecular weight substance having an anion or cation exchange group in a conventional manner. For example, an aqueous solution of the protease is passed through the ion exchange resin layer for modification thereof. This process is disclosed, for example, in "J. Am. Chem. Soc." Vol. 81, 4024 (1959) by M. A. Mitz et al.

The resultant water-insoluble modified protease is separated from unreacted protease, high molecular weight substance, and other water-soluble substances, if any, for example, by washing the reaction product with physiological saline till the washing shows no ninhydrin reaction and further washing with water to remove NaCl, followed by drying. Washing of the product can be conducted with an aqueous solution of other water-soluble metal salts such as sodium acetate or with water itself.

In the invention water-insoluble modified protease is used as an effective ingredient, since only such modified protease displays anti-inflammatory activity completely free from harmful side effects. When the modified protease is water-soluble, it exhibits undesired harmful effect due to absorption from intestine, failing to attain the object of the invention.

The water-insoluble modified protease is made into various forms in usual method for administration as an anti-inflammatory composition. For example, it may be made into tablets, granules or powder by usual method. Alternatively, it may be prepared as an ointment. Depending upon the form of preparation, it may be administered intraperitoneally, orally or locally.

For a better understanding of the invention examples are given below.

Example 1

1 g of α-chymotrypsin crystals were dissolved in 100 ml of 0.05 molar concentration solution of sodium acetate and the pH of the solution was adjusted to 7.3. To the solution was added dropwise with stirring a solution, cooled to 2°C, of 4 g of N-carboxy-γ-methyl-L-glutamic anhydride dissolved in 40 ml of dioxane. The mixture was stirred at 2°C for 30 minutes and left to stand at 4°C for 20 hours. The precipitate produced was separated by centrifuging at 20,000 r.p.m. at 0°C and washed with 0.05 molar concentration solution of sodium acetate. The washed precipitate showing no ninhydrin reaction was freeze-dried to produce 2.1 g of poly-γ-methyl - L - glutamyl - α - chymotrypsin in the form of white powder. The product was water-insoluble and contained 19.1 weight percent of α-chymotrypsin bonded with N-carboxy-γ-methyl-L-glutamic anhydride.

The esterolytic activity on N-acetyl-L-tyrosine ethyl ester of the resultant product was reduced to 0.15 time that of the starting α-chymotrypsin. Thus, it was evident from the content of chymotrypsin in the product that 78.6% of the activity was retained.

The poly - γ - methyl - L - glutamyl - α - chymotrypsin thus obtained and the starting α-chymotrypsin were respectively dispersed in 5 ml of physiological saline in such an amount as to produce esterolytic activity of 300 m M/min on N-acetyl-L-tyrosine ethyl ester by Hesterin method, whereby sample (I) containing the starting protease and sample (II) containing the resultant water-insoluble protease were obtained.

Each sample was intraperitoneally administered to rats in a dose of 5 ml/kg. In 30 minutes 0.05 ml of 1 wt.% carrageenin was subcutaneously injected in the sole of rear foot of each rat. Increase of volume of the rear foot was measured in 3 hours thereafter to determine inhibitive effect on edema with the results shown in Table 1 below, in which are also shown as a control the test results obtained by using physiological saline in the same manner as above.

TABLE 1

Sample Nos.	Inhibitive effect on edema	Result of autopsy
I	62%	Bleeding observed
II	65%	No change observed
Control	0	"

Example 2

5 ml of protease obtained from Bacillus sp 0—20 (deposited at Fermentation Research Institute of Agency of Industrial Science and Technology, Japan, since Feb. 20, 1969, with Deposition No. of FERM-P 270) was dissolved in 4.5 ml of 0.1 molar concentration of borate buffer containing 0.01 M $CaCl_2$ and having pH of 8.0. The protease used in this Example had a proteolytic activity of 4,000,000[PU]/g, which shows enzymatic activity of the protease producing non-protein substance showing folin colour corresponding to 1 γ of tyrosine in one minute as one unit

of enzymatic activity. To the solution was added 1 ml of succinyl anhydride dissolved in 0.5 ml of dioxane and the mixture was stirred for 30 minutes. The reaction mixture was dialyzed with 0.005 M of $CaCl_2$ for a day and the dialyzed substance was adsorbed to "DEAE-Sephadex" (Trade mark, diethylaminoethyl dextran of Pharmacia Fine Chemicals, Sweden) which had been buffered with 0.01 molar concentration of borate buffer containing 0.05 M of $NaCl_2$ at pH 8.0 and filled in a column. The resultant product was washed with physiological saline till the washing showed no enzymatic activity nor ninhydrin reaction. The product was further washed with water containing 0.001 M of $CaCl_2$ to remove sodium chloride and dried in vacuo at 4°C, whereby water-insoluble modified protease was obtained in the form of white powder. The bonded protease in the resultant modified protease was 6 wt.%.

The starting protease and the modified protease was compared with respect to proteolytic activity and esterolytic activity as follows. The esterolytic activity is expressed in terms of a value when each protease was used in an amount required for exhibiting a proteolytic activity of 100.

TABLE 2

Protease	Proteolytic activity on casein *1	Esterolytic activity on N-acetyl-L-tyrosine ethyl ester *2
Starting protease	100	100
Modified protease	100	1,500

Note: *1 Casein-Folin method
*2 Hesterin method

The starting protease and the modified protease were respectively dispersed in 5 ml of physiological saline in such an amount as to produce esterolytic activity on acetyl-tyrosine ethyl ester of 500 m M/min measured by Hesterin method, whereby samples (III) and (IV) were obtained.

Inhibition effect on edema of each sample was tested in the same manner as in Example 1 with the results shown in Table 3 below.

TABLE 3

Sample Nos.	Inhibition effect on edema	Result of autopsy
III	71%	Bleeding observed
IV	70%	No change observed

Example 3

"Nagase" (Trade mark, protease obtained from Bacillus subtilis, product of Nagase Sangyo Co., Ltd., Osaka, Japan) was subjected to succinylation and adsorbed to "DEAE-Sephadex" (Trade mark, the same as in Example 2) to produce water-insoluble modified protease in the same manner as in Example 2.

The proteolytic activity on casein and esterolytic activity on N-acetyl-L-tyrosine ethyl ester of the modified protease were respectively reduced to 1/10 and 8/10 times those of the starting protease.

The starting protease and the resultant modified protease were added to physiological saline in the same manner as in Example 1 to produce samples V and VI and tested in the same manner as in Example 1 with the results shown in Table 4 below.

TABLE 4

Sample Nos.	Inhibitive effect on edema	Result of autopsy
V	63%	Bleeding observed
VI	62%	No change observed

Example 4

In 75 ml of 1/15 molar concentration of acetate buffer containing 0.01 M of calcium acetate and having pH of 7.2 was dissolved at 4°C 481 γ of protease having proteolytic activity on casein of 4,000,000 [PU]/g obtained from Bacillus sp 0—20 (the same as

specified in Example 2). To the solution was added slowly with stirring 4 mg of N-carboxy-γ-methyl-L-tyrosine anhydride dissolved in 30 ml of dioxane and the surface of the mixture was covered with a small amount of toluene. Thereafter the mixture was left to stand at 4°C for 20 hours and the precipitate produced was separated by centrifuging at 10,000 r.p.m. at 0°C to produce water-insoluble modified protease.

Enzymatic activities of the starting protease and the resultant modified protease are shown in Table 5 below, in which esterolytic activity is expressed in terms of a value when each protease was used in an amount required for exhibiting a proteolytic activity of 100.

TABLE 5

Protease	Proteolytic activity on casein	Esterolytic activity on acetyl-tyrosine ethyl ester
Starting protease	100	100
Modified protease	100	1,570

The starting protease and the modified protease were respectively added to 5 ml of physiological saline in such an amount as to produce esterolytic activity on N-acetyl-L-tyrosine ethyl ester of 300 m M/min measured by Hesterin method, whereby samples VII and VIII were obtained.

Inhibition effect on edema of each sample was tested in the same manner as in Example 1 with the results shown in Table 6 below.

TABLE 6

Sample Nos.	Inhibition effect on edema	Result of autopsy
VII	53%	Bleeding observed
VIII	54%	No change observed

Example 5

One g of trypsin was dissolved in 0.1 molar concentration of phosphate buffer having pH of 7.6 and containing 50 wt.% of $HgSO_4$. To 40 ml of the resultant solution was slowly added with stirring at 4°C 800 mg of N-carboxy-L-tyrosine anhydride dissolved in 18 ml of dioxane and the mixture was left for standing at 4°C for 16 hours. The precipitate thus produced was separated by centrifuging at 10,000 r.p.m. at 4°C. The resulting product was washed with physiological saline till the washing showed no ninhydrin reaction and further washed with water to remove sodium chloride. The washed product was then freeze-dried, whereby 775 mg of water-insoluble modified protease was obtained in the form of powder. The bonded trypsin in the modified protease was 10.4 wt.%.

Using the starting protease (trypsin) and the modified protease samples IX and X were prepared in the same manner as in Example 1. Inhibitive effect of each sample was tested in the same manner as in Example 1 with the results shown in Table 7 below.

TABLE 7

Sample Nos.	Inhibitive effect on edema	Result of autopsy
IX	54%	Bleeding observed
X	54%	No change observed

WHAT WE CLAIM IS:—

1. An anti-inflammatory composition which comprises a water-insoluble modified protease and an adjuvant, said water-insoluble modified protease being a protease chemically bonded with a high molecular weight substance, and said high molecular weight substance having a molecular weight of from 20,000 to 200,000 and being selected from the group consisting of amino acid polymers having a skeletal chain containing repetitive peptide bonds, polysaccharides, polyaminostyrenes and copolymers of ethylene and maleic acid or anhydride, there being no water soluble protease or water soluble modified pretease present.

2. The anti-inflammatory composition according to Claim 1, in which said high molecular weight substance is an amino acid polymer having a skeletal chain containing repetitive peptide bonds.

3. The anti-inflammatory composition according to Claim 2, in which said amino acid polymer is a copolymer of L-leucine and p-amino-DL-phenyl aniline, a polymer of N-carboxy-γ-methyl-glutamic anhydride, a polymer of N-carboxy-γ-methyl tyrosine anhydride or a polymer of N-carboxy tyrosine anhydride.

4. The anti-inflammatory composition according to Claim 1, in which said high molecular weight substance is a polysaccharide.

5. The anti-inflammatory composition according to Claim 4, in which said polysaccharide is cellulose, diethylaminoethyl cellulose, carboxymethyl cellulose, p-aminobenzyl cellulose, m-aminoanisole cellulose, m-aminobenzoyloxymethyl cellulose, 3-(p-amino-m-methylanilino - 5 - chloro) - 1 - triacetyno cellulose, carboxymethyl cellulose azide, cyanur cellulose, bromoacetyl cellulose or cellulose citrate.

6. The anti-inflammatory composition according to Claim 4, in which said polysaccharide is dextran, diethylaminoethyl dextran, carboxymethyl dextran or a reaction product of dextran and isocyanate.

7. The anti-inflammatory composition according to Claim 1, in which said protease is trypsin, pepsin, chymotrypsin or pancreatin.

8. The anti-inflammatory composition according to Claim 1, in which said protease is bromelin or papain.

9. The anti-inflammatory composition according to Claim 1, in which said protease is a protease from one microorganism selected from the group consisting of Aspergillus melleus, Aspergillus oryzae, Penicillium notatum, Rhizopus chinensis Mucor racemosus, Trametes sanguinea, Llodella subpileata, Bacillus subtilis, Pseudomonas myxogenes, Streptomyces griseus and Streptomyces fradiase.

10. The anti-inflammatory composition according to Claim 1, in which said protease is chymotrypsin, bromelin or a protease from Bacillus subtilis or Aspergillus melleus.

11. An anti-inflammatory composition as claimed in Claim 1 and substantially as described herein with reference to the Examples.

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